

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Brovelli et al
Serial No: 09/575,307
Filing Date: May 19, 2000
Examiner: Michael V. Meller
Group Art: 1654
For: ECHINACEA INDUCTION OF PHASE II ENZYMES


Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.131

We declare that:

1. We are co-inventors of the patent application identified above.
2. Prior to May 9, 2000, we had completed the invention as described and claimed in the subject application in this country as evidenced by the following:
 - a. the Invention Conception Record and accompanying attachments submitted to the Alticor Legal Department (formerly Amway Corporation) (attached as Exhibit 1);
 - b. Lab notebook pages from one inventor, Kari Truax, (attached as Exhibit 2).
3. The Invention Conception Record and its accompanying figures show that, prior to May 9, 2000, we conceived of and reduced to practice a procedure for inducing phase II enzymes with chloroform-soluble *Echinacea purpurea* fractions as described and claimed in the subject application.
4. Additionally, Kari Truax's lab notebook pages outline the steps she performed to reduce our invention to practice in the Nutrilite Laboratories at 19600 6th Street, Lakeview, CA 92567-8403. These steps conform with Figure 1 and Pages 6-7 of the subject application and further support that, prior to May 9, 2000, we conceived of and reduced to practice, a procedure for inducing phase II enzymes with chloroform-soluble fractions of *Echinacea purpurea* as described and claimed in the subject application.
5. Each of the dates redacted from Exhibits 1 and 2 are prior to May 9, 2000.

6. We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.


Ernesto Brovelli, Ph.D

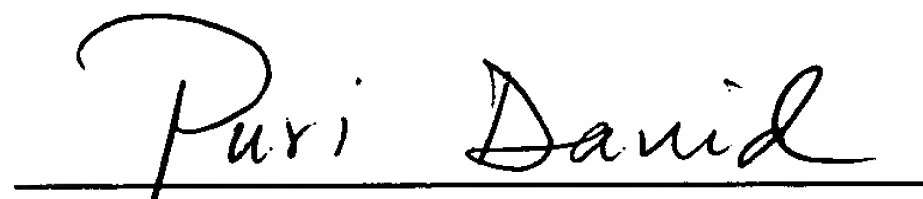
10-3-04
Date


Yingqin Li

10/4/04
Date

Kari Fitzgerald (maiden name - Truax)

Date


Puri David

10/3/04
Date

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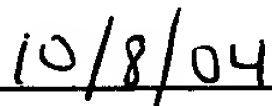
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Yingqin Li

Date



Kari Fitzgerald (maiden name – Truax)

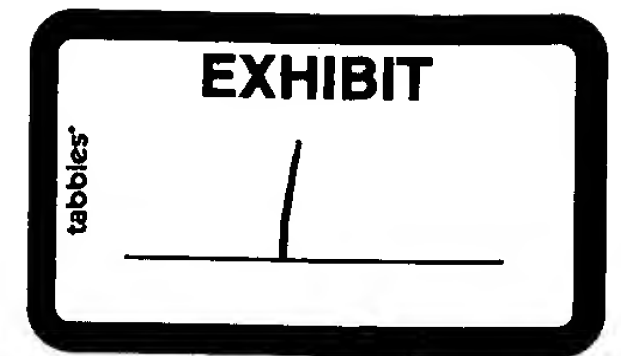


Date

Puri David

Date

**Amway Corporation
Invention Conception Record**



A. Title of your invention.

Phase II Enzyme Induction by *Echinacea* Extract and Its Fractions

B. Describe your invention including specific embodiments and alternatives, ranges and products, and process/apparatus variations. Include photographs, sketches, flow sheets, or other drawings where appropriate.

We have found that certain *Echinacea purpurea* extracts and some of its fractions induce the expression of Phase II enzyme. The extracts found to induce the expression of this enzyme are derived from both the aboveground organs and the roots.

On that basis, experimentation was further refined to determine the level of induction for *Echinacea purpurea* fractions extracted with solvents of different polarity. It was determined that for aboveground organs, it is the petroleum ether fraction that results in the greatest induction of phase II enzyme. For roots, it is the chloroform fraction that brings about maximum induction.

C. Describe what problem was solved and how you solved it. Describe how your invention differs from art you are aware of and describe the advantages of your invention.

To our knowledge, and based on an extensive literature review, there is no previous knowledge about this kind of mechanism in *Echinacea*.

The induction of phase II enzymes has extremely important implications for the human body. These enzymes have detoxifying activity of potential carcinogens.

In addition to *Echinacea*'s well-documented immunomodulatory functions, this finding can extend the activity of *Echinacea* as a cancer chemopreventive agent.

D. When did you first think of this invention?

On ^{redacted} we thought that the recently developed prototypes might have Phase II enzyme properties and decided to submit them to a bioassay.

E. What records do you have to document when you first thought of this invention?

Laboratory notebook.

F. To whom did you first disclose this invention?

Kha Tran

G. On what date did you make this disclosure?

redacted

H. When did you first do any actual experimental work concerning the invention?

redacted

I. Who has observed the progress of your experimental work?

Rodney Johnson & Kevin Gellenbeck.

J. Are there other ICRs now on file or contemplated, yours or others, that tie in closely with this invention?

No.

K. Has panel or consumer testing been conducted?

Yes _____ No. ___X___ If yes, when? Describe the testing.

- L. Are there any plans for consumer or panel testing?

Yes _____ No. ___X___ If yes, when? give details.

Not in the immediate plans.

- M. Give dates and details regarding any samples, sales, information, or publications relating to this invention which have been or will be given to persons outside Amway Corporation.

No information has been disclosed to outside parties.

- N. Are you aware of any pertinent literature or patent references? Give details.

None whatsoever.


- O. Is commercial use imminent? If yes, indicate the anticipated earliest date of commercial use.

The extracts are currently being manufactured by Nutrilite/Amway, although with the purpose of enhancing the immune system.

This is an extremely novel function for this herb and it would be advantageous to exploit this benefit commercially.

NOTE: IF ANY CHANGES TO THE ABOVE INFORMATION OCCUR,
ADVISE A MEMBER OF THE PATENT REVIEW TEAM IMMEDIATELY.

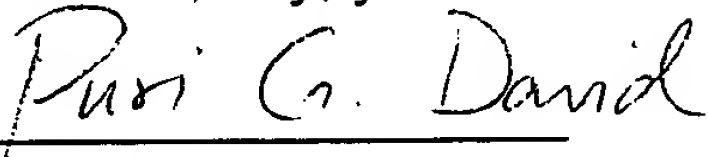
Inventor Ernesto A. Brovelli

Signed  redacted
month, day, year


Inventor Yong Quian

Signed _____
month, day, year

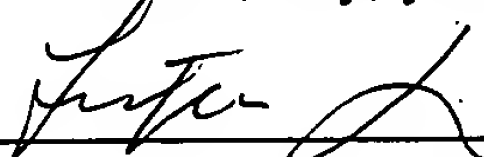
Inventor Puri G. David

Signed  redacted
month, day, year

Inventor Kari L. Truax

Signed  redacted
month, day, year

Inventor Yingqin Li

Signed  redacted
month, day, year

The foregoing signed disclosure was read and understood by me on the date
hereinafter set forth.

Witness 

Signed redacted
month, day, year

Witness _____

Signed _____
month, day, year

Extraction Protocol

100 g *Echinacea purpurea* ground roots or aerial parts (100 g)

80% Methanol

Roots: 11.7 g
Aerial parts: 20.5 g
(For the next extraction we carried over Roots: 5.8 g and Aerial parts: 10.3 g (100%))

Petroleum Ether

Roots: 0.1g (1.72%)
Aerial parts: 0.1582 g (1.55%)

Chloroform

Roots: 0.2383 g (4.11%)
Aerial parts: 0.2185 g (2.14%)

*Adjustment to pH 2

Chloroform

Roots: 0.0535 g (0.92%)
Aerial parts: 0.0442 g (0.43%)

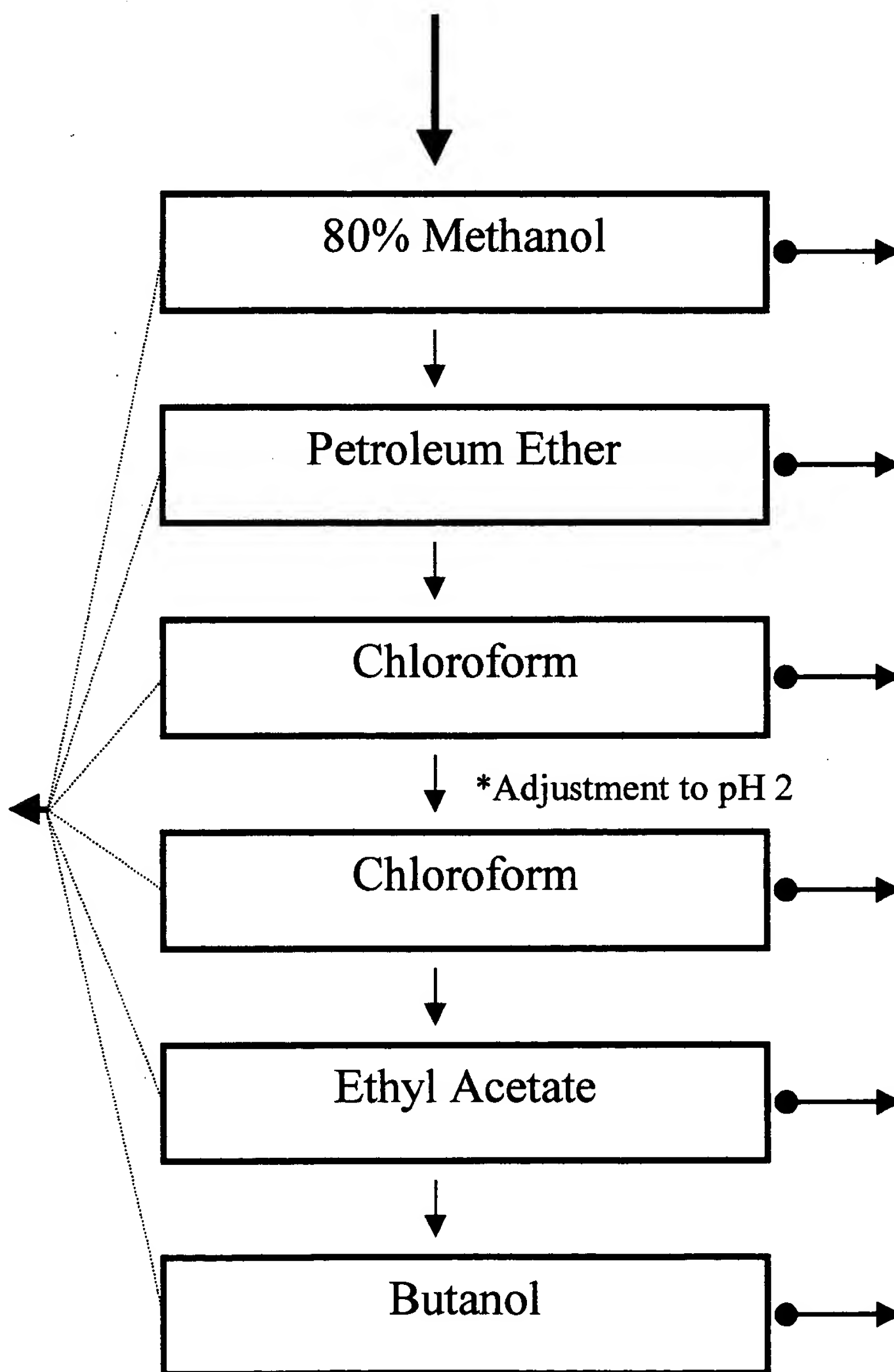
Ethyl Acetate

Roots: 0.6191 g (10.67%)
Aerial parts: 0.3295 g (3.23%)

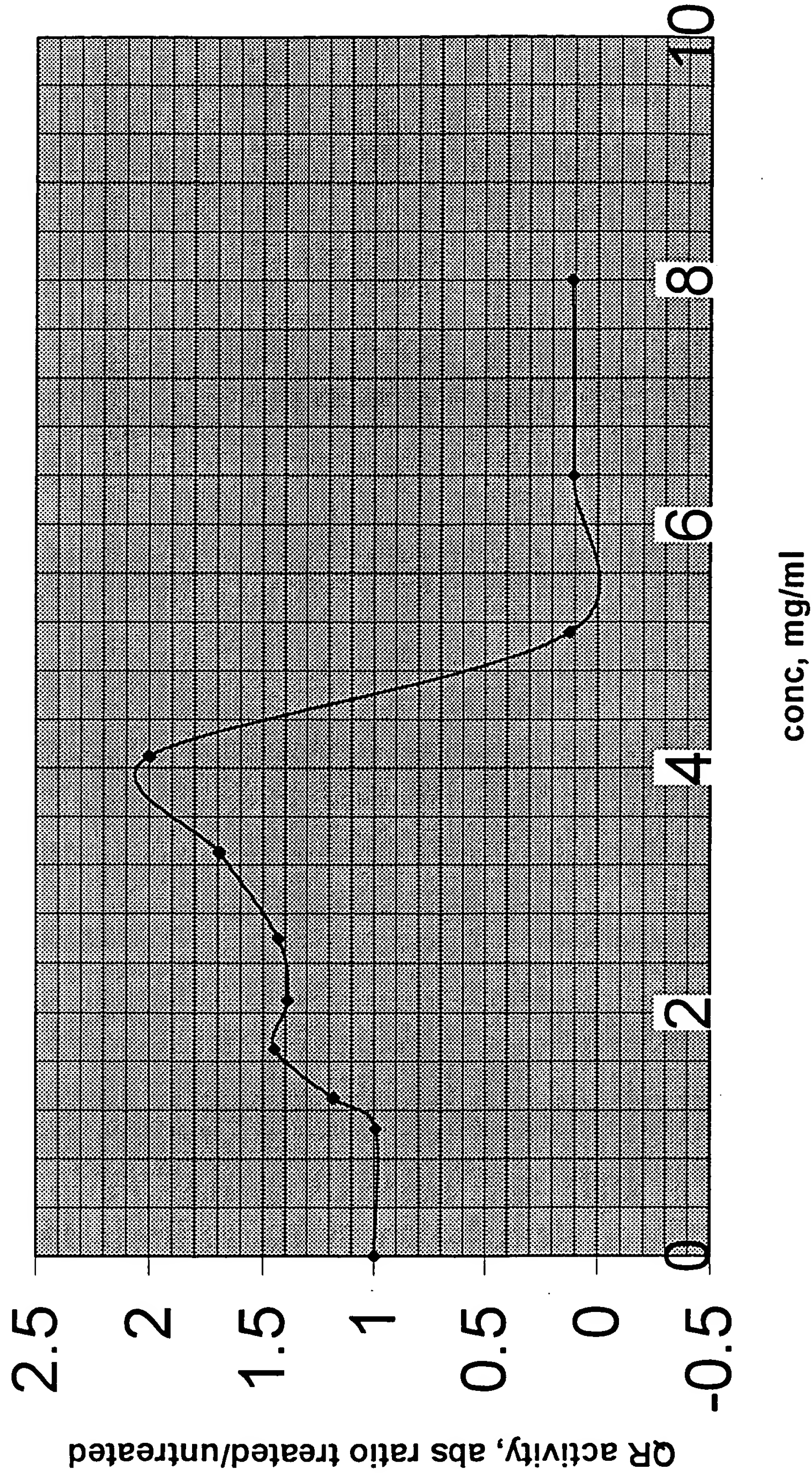
Butanol

Roots: 0.0607 g (1.05%)
Aerial parts: 0.0634 g (0.62%)

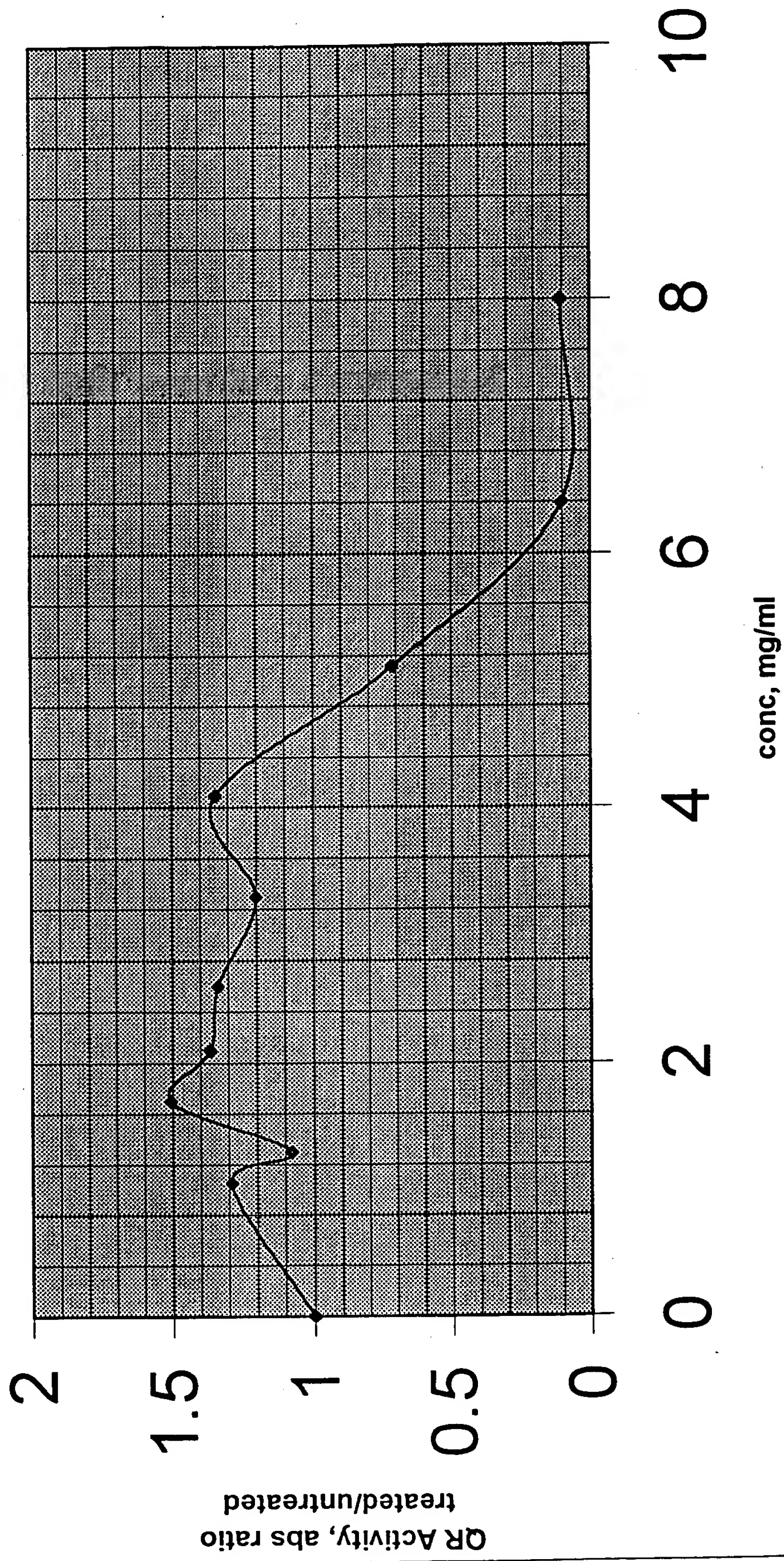
Quinone Reductase (QR) Activity



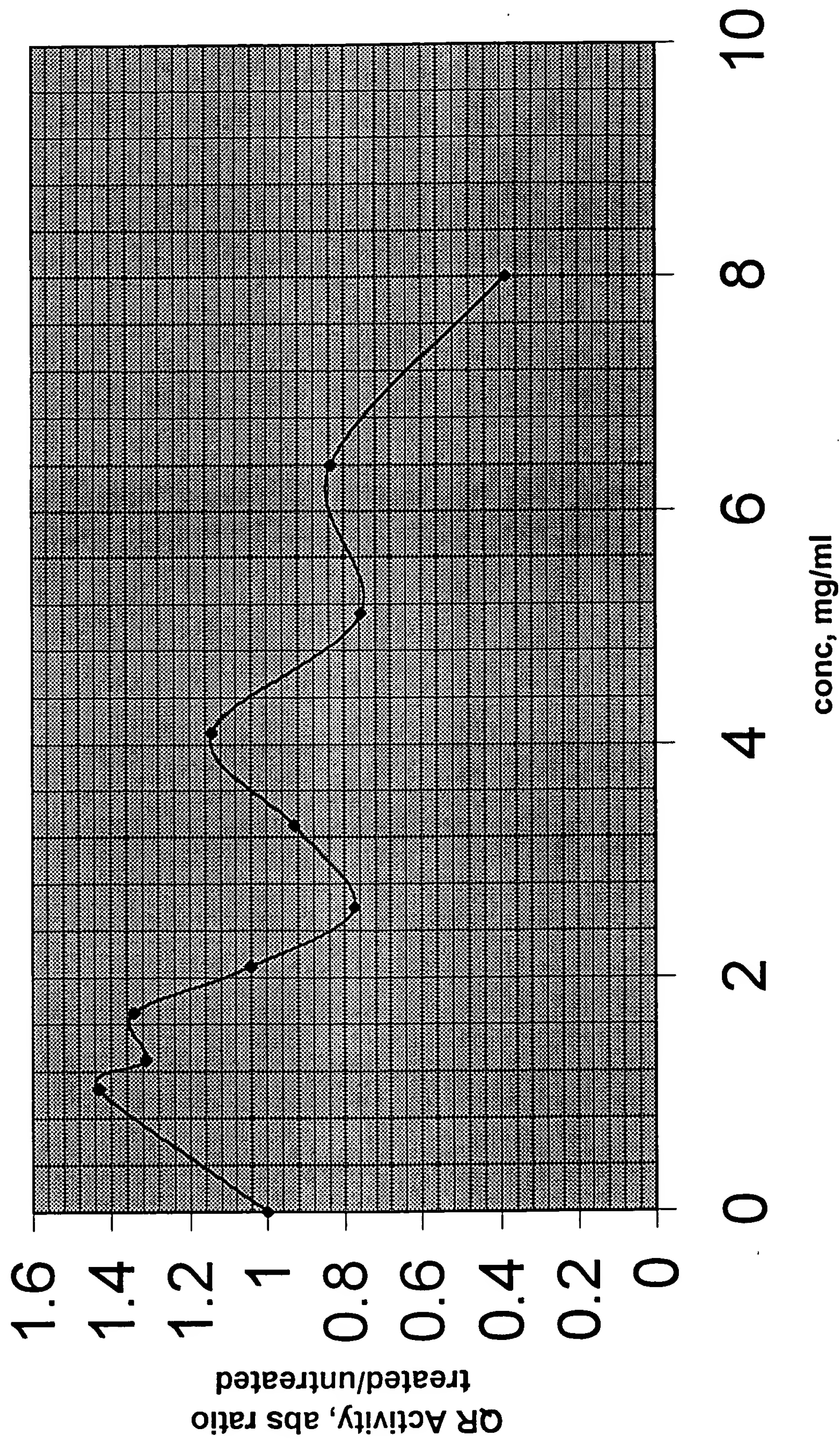
Echinacea purpurea root extract,
60 C extraction



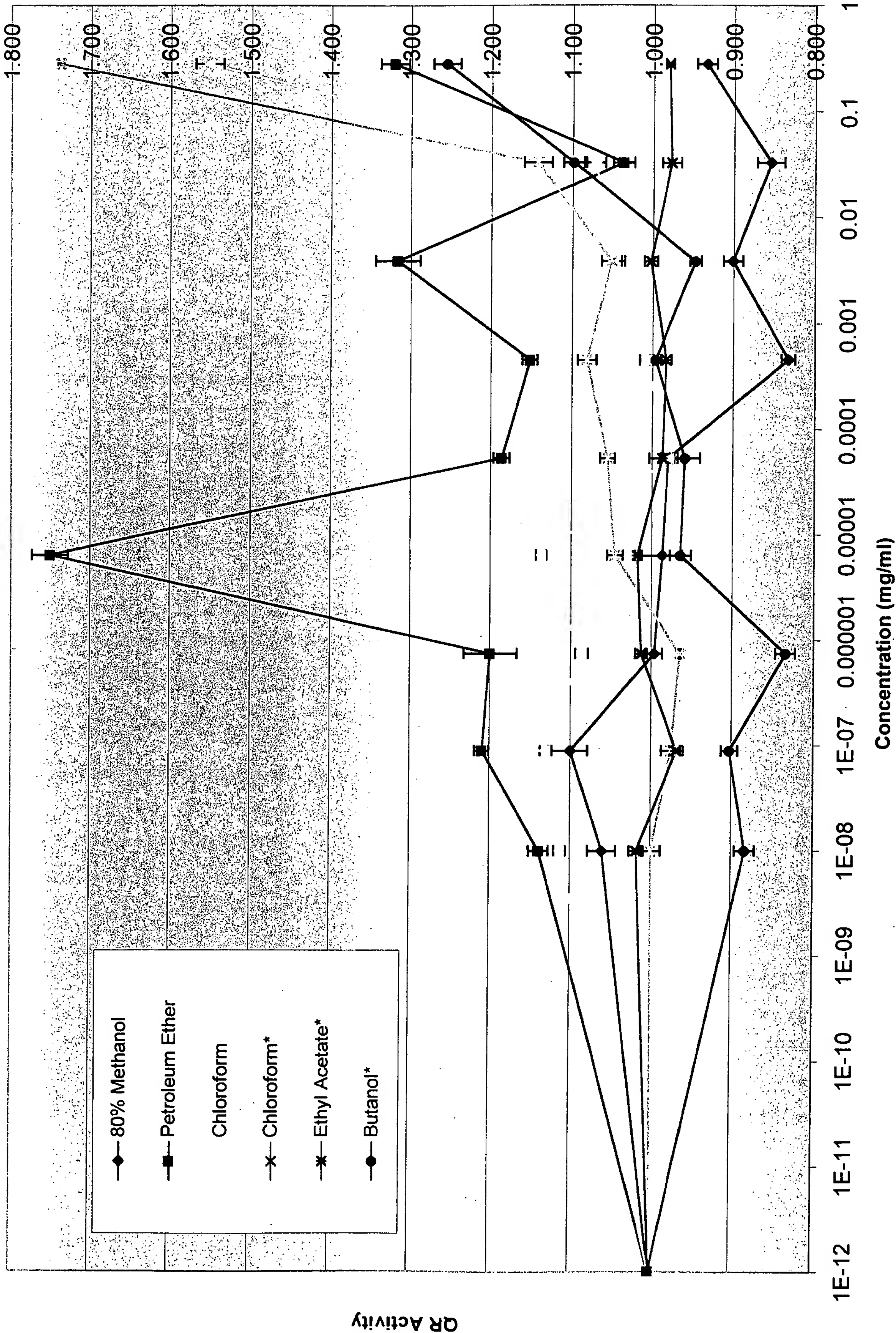
Echinacea purpurea root extract,
20 C extraction



Echinacea purpurea aerial parts extract

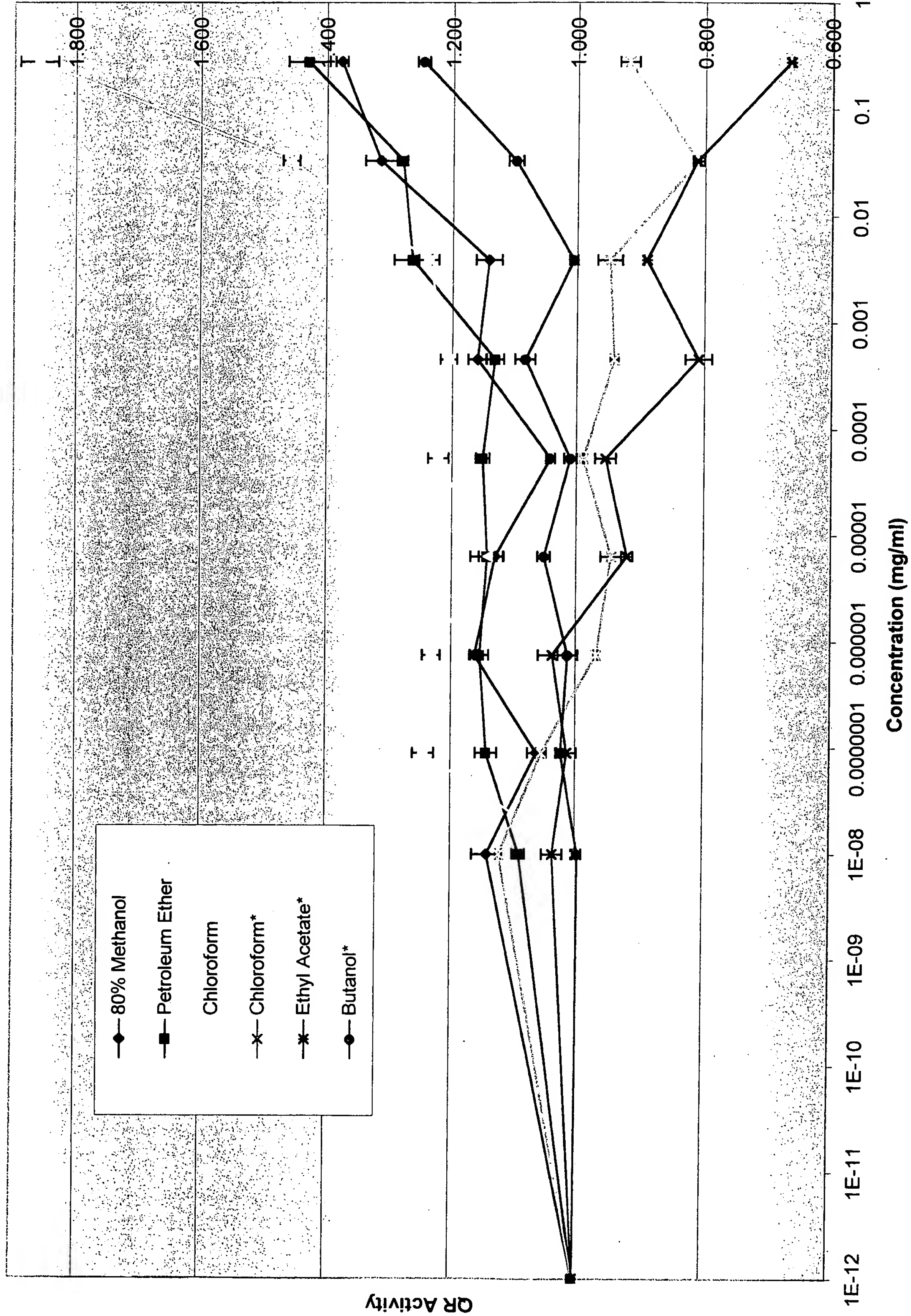


Induction of Quinone Reductase by *Echinacea purpurea* Aerial Fractions



* The aqueous residue was acidified to pH 2.

Induction of Quinone Reductase by *Echinacea purpurea* Root Fractions



* The aqueous residue was acidified to pH 2.

EXHIBIT

2

tabbies

Protocol is from:

~~Maffei-Facino, R., Carini, M.,~~~~(1995) Echmezon~~ Extract~~+ Caffeoyl conjugates~~

100g

Roots or Tops

by 80% MeOH twice

500ml x 2 times

① Condense 60min

② Condense 40min

Maffei-Facino, R., Carini, M.,

Aldini, G., et al. (1995)

Extract

Direct characterization of
caffeoyl esters w/anti-hyaluronidase
activity in crude extracts
from E. a. roots by fast atom
bombardment tandem mass
spectrometry. Farmaco 48(10)
1947-1961.

Dry ↓

evaporate

@ 30-40°C

Residue

R=11.7g

T=20.5g

add 100ml H₂O + sonicate until
homogenousResidue + H₂O

R=5.8g

T=10.2g

Fractionate (separatory funnel)

①

Petroleum Ether

100ml x 3

↓ Na₂SO₄

↓ evaporate

R=0.1g
1.72%
T=0.1582g
1.55%

②

Chloroform
Methyl Chloride

100ml x 3

↓ Na₂SO₄

↓ evaporate

R=0.2383g
4.11%
T=0.2185g
2.14%

③

Adjust pH
of H₂O to 2
using HCl

Fractionate

④

Methyl Chloride
(100ml x 3)↓ H₂O 50 x 2 wash↓ Na₂SO₄

↓ evaporate

T=0.0442
R=0.0535
0.92%
T=0.2185
0.43%
g

⑤

Ethyl Acetate
(100ml x 3)↓ H₂O 50 x 2 wash↓ Na₂SO₄

↓ evaporate

R=0.6191
10.67%
T=0.3295
3.23%
g

⑥

Butanol
(100ml x 3)↓ H₂O 50 x 2 wash↓ Na₂SO₄

↓ evaporate

R=0.0607g
0.607%
T=0.0634g
0.634%
g

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Day 2: Tuesday

Prepare 2 MEM (w 10% calf serum + 1% antibiotic)

Prepare the samples:

#1 Brassica concentrate

$$\begin{aligned} & 100\text{mg sample} / 10\text{ml 2 MEM (w/calf serum + antibiotic)} \\ & = \underline{10\text{mg/ml}} \end{aligned}$$

#2 E. p. Roots

$$\begin{aligned} & - 50\text{mg sample} / 10\text{ml 2 MEM (w/calf serum + antibiotic)} \\ & = \underline{5\text{mg/ml}} \end{aligned}$$

$$\begin{aligned} & - 100\text{mg sample} / 10\text{ml 2 MEM (w/ " ")} \\ & = \underline{10\text{mg/ml}} \end{aligned}$$

#3 E. p. Tops

$$\begin{aligned} & - 50\text{mg sample} / 10\text{ml 2 MEM (w/ " ")} \\ & = \underline{5\text{mg/ml}} \end{aligned}$$

$$\begin{aligned} & - 100\text{mg sample} / 10\text{ml 2 MEM (w/ " ")} \\ & = \underline{10\text{mg/ml}} \end{aligned}$$

#4 E. p. Concentrate

$$\begin{aligned} & 50\text{mg sample} / 10\text{ml 2 MEM (w/ " ")} \\ & = \underline{5\text{mg/ml}} \end{aligned}$$

#5 E. p. Concentrate

$$\begin{aligned} & 100\text{mg sample} / 10\text{ml 2 MEM (w/ " ")} \\ & = \underline{10\text{mg/ml}} \end{aligned}$$

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- Vortex all of the samples for ≈ 2 minutes
- Then centrifuge @ speed 6 for ≈ 10 minutes
(TEC Clinical centrifuge)

Treatment w/ samples:

- 1.) Remove all of the old α MEM medium from the 96 well plates.
- 2.) To lanes 1+2 add 300ul α MEM medium, to lanes 3-12 add 150ul α MEM medium.
- 3.) Add 100ul sample to lane 12, pipet up + down 4 times, remove 100ul from lane 12 + put into lane 11, pipet up + down 4 times, remove 100ul from lane 11 + put into lane 10 pipet up + down 4 times... continue this through lane 3 @ lane 3 once all is mixed (4 times) remove 100ul + discard.
- 4.) Repeat step #3 using 100ul of new sample.
- 5.) Add 150ul of α MEM medium to lanes 3-12, to make all wells have a final volume of 300ul.

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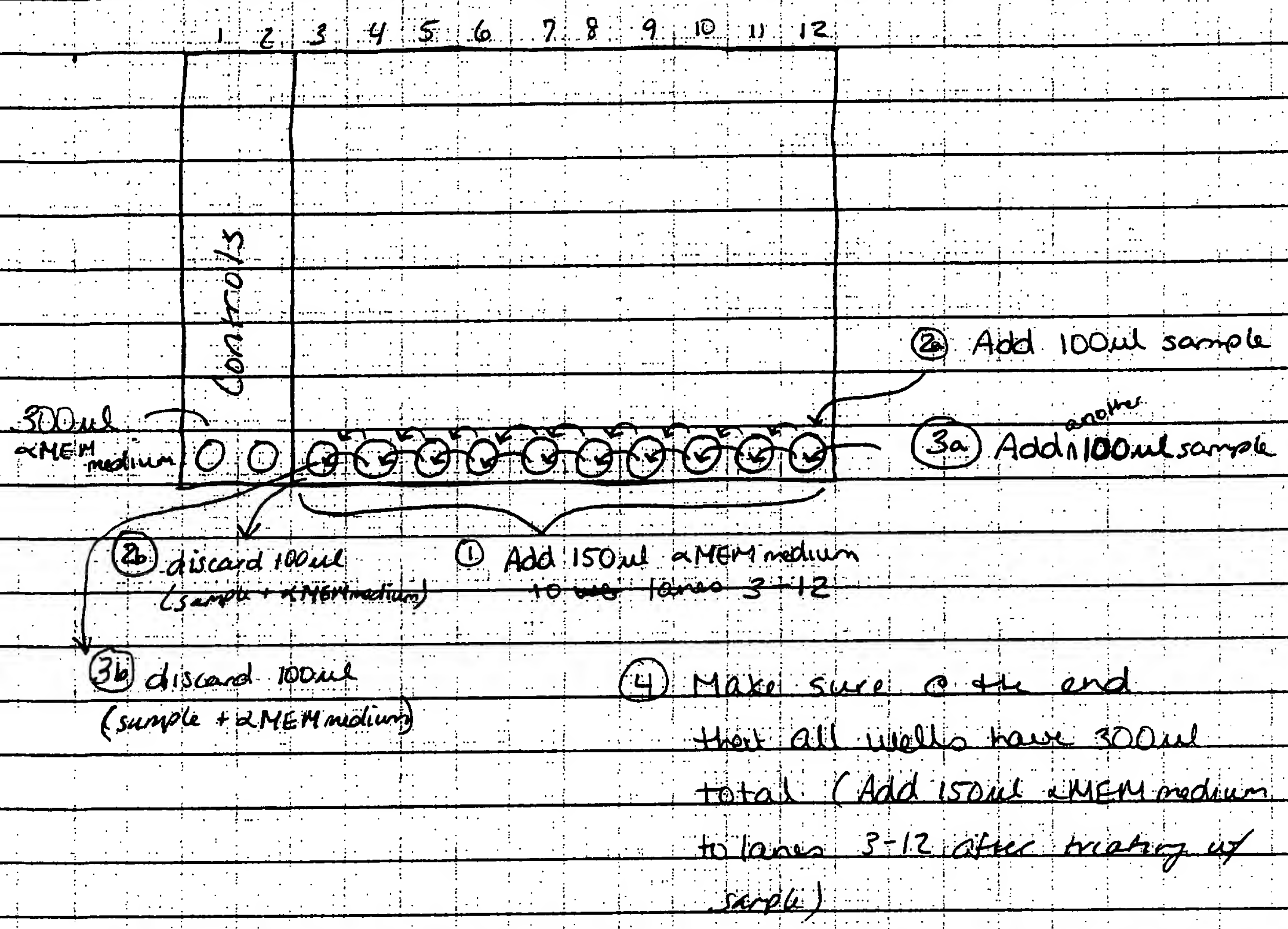
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6.) Incubate @ 37°C for 48 hrs.

96 well plate:



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Plate #1: Brassica Concentrate (10mg/ml)

923 bracc dat	controls	10mg/ml

- capture image

save as 92399br.cpf

Plate #2: E. purpurea Roots (5 + 10mg/ml)

923 eproots.dat	controls	5mg/ml
		10mg/ml

92399er.cpf

Plate #3: E. purpurea Tops (5 + 10mg/ml)

923 eptops.dat	controls	5mg/ml
		10mg/ml

92399et.cpf

Plate #4: E. purpurea Concentrate (5mg/ml)

923 epc50.dat	controls	5mg/ml

92399ec5.cpf

Plate #5: E. p. Concentrate (10mg/ml)

923 epc100	controls	10mg/ml

92399ec1.cpf

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Day 4 : PII Enzyme Assay - Reading

Day 3 : Wednesday - I was sick, did not need to prepare cocktail, it was prepared last time + stored in freezer.

Day 4 : Thursday - Reading + Evaluation of results

- 1.) Look @ wells under microscope. Note if any of the concentrations caused cell death.
* do this b/4 decanting wells + adding Rxn cocktail

Plate #1 : Brassica concentrate (10mg/ml)

Lane 9 - start to see dark cells - maybe dead
but when I move the plate they don't move
↓
keep seeing more + more through lane 12
Lane 12 :

Plate #2 : E. purpurea roots

5mg/ml

Lane 12 see some dark cells same as brassica concentrate
other lanes look fine

10mg/ml

Lane 9 start to see dark cells
↓
keep seeing more + more
Lane 12

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Plate #3

E. purpurea Tops

5mg/ml

Lane 10 - start to see dark cells



Lane 12 - ↑ dark cells

10mg/ml

Lane 2 - start to see dark cells



Lane 12 - ↑ dark cells, still not that much

Plate #4

E. purpurea concentrate (5mg/ml)

- see a lot of the cells grouped together - dark brown -
- do not look attached but when you shake the wells
- they don't move, also see attached cells



cells brown (don't appear to be floating)

also see attached cells

Plate #5

E. purpurea concentrate (10mg/ml)

- same as plate appear the same as plate #4

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Day 4: PII Enzyme Assay

Cocktail - thaw, before assay add 100 units
glucose 6-phosphate dehydrogenase + 50 ul
50mM Menadione in acetonitrile per 50ul cocktail
(previously prepared)

Enzyme Assay:

- 1.) Wash wells as completely as possible
- 2.) Add 30ul of 0.8% digitonin in 2mM EDTA
(pH 7.2) to all wells + incubate 10min
@ 37°C to lyse cells. Then 10min
(@ 75°C) w/ 100rpm shaking (Orbital
Shaker) to release enzymes
⇒ Orbital Shaker setting @ 4
- 3.) Add 100ul cocktail + start timing
(2-10min) * 5 minutes this time *
- 4.) Stop the rxn by adding 30ul
of 0.3mM dicoumarol in 0.5%
DMSO + 5mM KH_2PO_4 (pH 7.4)
- 5.) Read plate @ 610nm + record OD values
using microplate plate reader.

Evaluate Data next week

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Cocktail: ~~draw~~ before assay add 100 units
glucose 6 phosphate dehydrogenase and 50ul
of 50mM Menadione in acetone buffer
⇒ per 50ml aliquot of previously prepared
cocktail

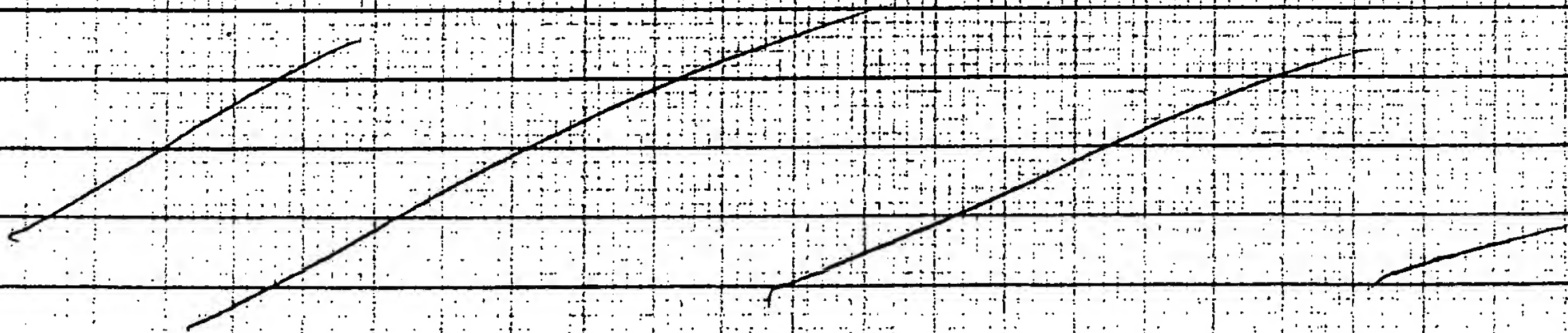
Enzyme Assay

Follow the steps on pg 77

Evaluate Data:

Record Appearance of wells

See notebook # 7104



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#9 Chloroform pH.8 Roots (20mg/ml)

10799^{cr2} ERZ

Control:

Mean OD = 0.671 Std Dev = 0.043 CN = 6.342

20ul	3.65e-11	5.44e-10	8.12e-9	1.21e-7	1.81e-6	2.7e-5	4.03e-4	0.006	0.0898	1.34
Conc (mg/ml)	2e-9	2e-8	2e-7	2e-6	2e-5	2e-4	2e-3	2e-2	0.2	2
Mean OD	0.753	0.707	0.650	0.635	0.664	0.632	0.637	0.544	0.617	0.242
Std Dev	0.020	0.031	0.014	0.064	0.004	0.026	0.078	0.037	0.060	0.084
CN	2.620	4.334	2.165	10.10	0.643	4.165	12.25	6.726	9.754	34.85
QR Activity	1.122	1.054	0.969	0.946	0.990	0.942	0.949	0.811	0.994	0.361
									0.920	

20ul	2e-9	2e-8	2e-7	2e-6	2e-5	2e-4	2e-3	2e-2	0.2	2
Conc (mg/ml)	3.65e-11	5.44e-10	8.12e-9	1.21e-7	1.81e-6	2.7e-5	4.03e-4	0.00602	0.0898	1.34
Mean OD	0.663	0.635	0.571	0.595	0.627	0.557	0.602	0.611	0.606	0.275
Std Dev	0.064	0.051	0.077	0.038	0.064	0.040	0.041	0.042	0.049	0.035
CN	9.596	8.111	13.40	6.353	10.27	7.252	6.877	6.912	8.162	12.79
QR Activity	0.988	0.946	0.851	0.887	0.934	0.830	0.877	0.911	0.903	0.410

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#10 Chloroform pH2 Tops (20mg/ml)

10799 ct 2

Control:

Mean OD = 0.610

Std Dev = 0.049

CV = 8.081

<u>20ul</u>	$3.65e^{-11}$	$5.44e^{-10}$	$8.12e^{-9}$	$1.21e^{-7}$	$1.81e^{-6}$	$2.7e^{-5}$	$4.03e^{-4}$	0.006	0.0898	1.34
Conc (mg/ml)	$2e^{-9}$	$2e^{-8}$	$2e^{-7}$	$2e^{-6}$	$2e^{-5}$	$2e^{-4}$	$2e^{-3}$	$2e^{-2}$	$2e^{-1}$	2
Mean OD	0.610	0.595	0.588	0.638	0.644	0.660	0.642	0.697	1.061	0.286
Std Dev	0.048	0.049	0.006	0.040	0.038	0.046	0.050	0.070	0.014	0.039
CV	7.807	8.234	0.935	6.338	5.932	7.030	7.798	10.05	1.282	13.77
GR Activity	1.0	0.975	0.964	1.046	1.056	1.082	1.052	1.143	1.739	0.469

<u>30ul</u>	$2e^{-9}$	$2e^{-8}$	$2e^{-7}$	$2e^{-6}$	$2e^{-5}$	$2e^{-4}$	$2e^{-3}$	$2e^{-2}$	0.2	2
Conc (mg/ml)	$3.65e^{-11}$	$5.44e^{-10}$	$8.12e^{-9}$	$1.21e^{-7}$	$1.81e^{-6}$	$2.7e^{-5}$	$4.03e^{-4}$	0.00602	0.0898	1.34
Mean OD	0.611	0.642	0.618	0.638	0.623	0.679	0.712	0.737	0.961	0.380
Std Dev	0.047	0.049	0.021	0.026	0.058	0.030	0.062	0.038	0.101	0.006
CV	7.733	7.620	3.357	4.110	9.312	4.484	8.648	5.196	10.49	1.493
GR Activity	1.002	1.052	1.013	1.046	1.021	1.113	1.167	1.208	1.575	0.623

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